A novel method for the *In vitro* evolution of aptamers and applications in protein detection and purification

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Abstract

One of the key components of proteomics initiatives is the production of high affinity ligands or probes that specifically recognize protein targets in assays that detect and capture proteins of interest. One particularly versatile set of probes with tremendous potential for use as affinity molecules are aptamers. Aptamers are short single stranded DNA or RNA sequences that are selected *in vitro* based on affinity for a target molecule. Aptamers offer advantages over traditional antibody-based affinity molecules in their ease of production, regeneration, and stability, largely due to the chemical properties of nucleic acids versus amino acids. We describe an improved *in vitro* selection protocol that relies on magnetic separations for DNA aptamer production that is relatively easy and scalable without the need for expensive robotics. We demonstrate the ability of aptamers that recognize thyroid transcription factor (TTF1) to bind their target protein with high affinity and specificity, and detail their uses in a number of assays. The TTF1 aptamers were characterized using surface plasmon resonance, and shown to be useful for enzyme-linked assays, Western blots, and affinity purification.

Introduction

The future success of proteomics depends on its ability to follow in the footsteps of genomics, where the development of new technologies generated an abundance of sequence data enabling researchers to probe problems that relate to the entire nucleic acid component of the cell. For the promise of proteomics to be realized, new tools are needed that will enable large-scale investigations of protein structure, function, and interactions. Significant progress has been made in proteomic technology development in many areas (1), including high-throughput gene cloning (2), protein production (3, 4), mass spectrometry (5), 2-D PAGE (6), and microfluidics to allow large-scale proteomics to proceed. One important set of tools that has been improved with moderate success are affinity reagents that function as antibodies to serve as protein probes. Affinity molecules that specifically bind proteins of interest can detect bound proteins in a protein microarray, or capture protein complexes for functional identification (7). Often these molecules can alter biological activity due to their binding and inhibit critical interactions by sterically blocking access to active sites and interaction surfaces, and thus present an opportunity to serve as functional probes as well as therapeutics. Traditionally, antibodies have satisfied the demand for such ligands, however as recombinant protein production gains throughput and pharmaceutical target repertoires expand, the ability to efficiently generate antibodies quickly falls short.

Several alternatives to antibodies have been investigated, such as single chain antibodies (scFv) (8), peptides displayed on protein domain scaffolded surfaces (9), peptides, and peptoids (synthetic peptides) (10). Each of these alternatives has drawbacks that limit their uses, such as problems of stability in varying conditions (ionic strength, temperature, and pH) and of low affinity, making some antibody alternatives ineffective for detecting proteins under many conditions (1).

The idea of using single stranded nucleic acids (aptamers) as affinity molecules for proteins, first described in 1990 (11, 12, 13), has shown modest progress. The concept is based on the ability of short (20-80 mer) sequences to fold, in the presence

of a target, into unique 3-dimensional structures that bind the target with high affinity and specificity. Aptamers are generated by a process that combines combinatorial chemistry with *in vitro* evolution, known as SELEX (Systematic Evolution of Ligands by Exponential Enrichment). Following the incubation of a protein with a library of DNA or RNA sequences, typically 10^{14} molecules in complexity, protein-DNA complexes are isolated, the DNA is amplified, and the process is repeated until the sample is enriched with sequences that display high affinity for the protein of interest. Since the selection pressure for this *in vitro* method of evolution is high affinity for the target, aptamers with low nanomolar affinities are often obtained. Aptamers offer advantages over protein-based affinity reagents due to the nature of nucleic acids, which provides for increased stability, ease of regeneration (PCR or oligonucleotide synthesis), and simple modification for detection and immobilization.

Although SELEX appears to be technically very simple, small alterations to a protocol can have a large impact on the success of generating aptamers. Perhaps this explains why thirteen years since its first citation in the literature, only approximately forty protein-detecting aptamer sequences have been published, and very few have been well characterized. Although high-throughput methods for aptamer production have been published, most require expensive robotics and have not produced aptamers against large numbers of diverse targets (14). Of course, in order for aptamer production to be truly high-throughput, a supply of purified proteins must also be available as targets.

In this report, we describe an improved protocol for DNA aptamer production that is relatively easy and scalable without the need for expensive robotics. In addition, we fully demonstrate the abilities of our aptamers to bind their target protein with high affinity and specificity, and detail their uses in a number of assays. As a target, we use thyroid transcription factor 1 (TTF1), a well characterized member of the NK homeodomain transcription factors (15, 16). TTF1 is expressed in the developing thyroid, lung, and brain of vertebrates, and several effector genes have been identified in thyroid and lung tissues (16). The DNA recognition site of TTF1 differs from other homeodomain containing proteins, attributed to the NK-type homeodomain (18). Following 15 rounds of selection, we characterized the affinity and specificity of several aptamers, and describe their uses in assays for the capture and identification of proteins, such as Western blots, enzyme-linked assays, and affinity purification.

Materials and Methods

Cloning, protein expression, and purification. TTF1 was cloned from *Ciona intestinalis* 16 hour embryos following RNA isolation (Trizol reagent, Gibco) and first strand synthesis (Superscript First Strand Synthesis System for RTPCR, Gibco) by PCR using the following gene specific primers that contained 5' Ligation Independent Cloning (LIC) (Novagen) compatible ends: Forward 5'-GGTATTGAGGGTCGCTCAGTTAGCCCAAAGCATTCG-3'; Reverse 5'-AGAGGAGAGTTAGAGCCTTATCGGTAAACACTGTACAGGATCG-3'. LIC was performed as previously described to insert the coding sequence of *Ciona intestinalis* TTF-1 into the vector pNHis, which adds a hexahistidine tag to the amino-terminus of the encoded protein (4).

After screening several expression conditions, TTF1 was found to be most highly expressed in Rosetta (DE3) pLysS cells (Novagen) induced with 1.0 mM IPTG at 37 ^{o}C for 4 hours. One liter of culture grown under these conditions was harvested by centrifugation at 5,000 x g for 10 min at 4 ^{o}C. The pellet was resuspended in 30 mL of resuspension buffer (50 mM Na_2HPO_4, 300 mM NaCl, 20 mM imidazole, 0.1% Triton X100, 5 mM 2-mercaptoethanol at pH 7.0 with 1 mM PMSF Plus (Roche), and 1x Protease Inhibitor Cocktail (Sigma)). The cells were then lysed with the Emulsiflex–C5 (Avestin) homogenizer at 15,000 psi. The lysate was centrifuged at 15,000xg for 25 min at 4 ^{o}C to remove insoluble material. Chelating Sepharose High Performance resin (Amersham) was charged with 0.1 M $NiSO_4$ and washed with 10 column volumes of sterile water. The cleared lysate was incubated with 300 μL of 50% slurry of Ni^{+2} charged resin and bound in batch for 20 min with constant rotation then loaded onto an empty polypropylene column (Qiagen) and allowed to drain by gravity flow. The 150 μL column was then washed with 50 column volumes of resuspension buffer containing 20% glycerol and the bound proteins were eluted with 0.5 column volumes of elution buffer (1 M imidazole, 50 mM Na_2HPO_4, pH 7.0, 150 mM NaCl, 0.1% Triton-X100, 20% glycerol, and 5 mM 2-mercaptoethanol). The concentration and purity were determined using 4 μL in a Protein 200 Lab-chip kit (Caliper) run on an Agilent 2100 Bioanalyzer.

In vitro selection of aptamers. A degenerate oligonucleotide library was synthesized at 1 μmole scale and HPLC purified (Operon). This material was diluted to 0.1 nmol/μL in 10 mM Tris, pH 8, and stored at -20 ^{o}C. This library, referred to as "LICSelexApt", is composed of 40 random nucleotides flanked by sequences suitable for ligation independent cloning (LIC): 5'-GGTATTGAGGGTCGCATC-40N-GATGGCTCTAACTCTCCTCT-3'. Primers that anneal to the 5' and 3' sequences flanking the degenerate region of LICSelexApt that were used during the selection and cloning were: "LICSelexF": 5'-GGTATTGAGGGTCGCATC-3'; "LICSelexR": 5'-AGAGGAGAGTTAGAGCCATC-3'; in biotinylated and non-biotinylated forms (HPLC purified, Operon).

Protein-bound Ni-NTA magnetic beads were prepared by first equilibrating 150 μL of a 5% slurry (approximately 45 μg capacity) of Ni-NTA magnetic beads (Qiagen) into PBS-T (50 mM K_2HPO_4, pH 7.5, 150 mM NaCl, 0.05% Tween 20). The equilibrated beads were resuspended in 1250 μL of PBS-T and 25 μL of 2 mg/mL purified TTF1 was added (a 1:50 dilution to lower the imidazole concentration) and mixed with rotation for 30 min at 4 ^{o}C. The bead-bound TTF1 was then washed 3x with 1 mL PBS-T, and diluted to 0.25 μg/μL (5 pmol/μL of 50 kDa TTF1) with PBS-T and stored at 4 ^{o}C.

In the initial round of selection, the "LICSelexApt" library was incubated with the bead-bound TTF1 using a 10-fold molar excess of ssDNA in a volume that gave a 10 nM TTF1 concentration. 1 nmol of "LICSelexApt" was diluted into 100 μL of PBS-T in a PCR tube and heated to 95 ^{o}C for 2 min then immediately cooled at 4 ^{o}C. This material was added to 10 mL of PBS-T containing 1 μg/mL BSA, 0.1 μg/mL dIdC. 100 pmol of bead-bound TTF1 was then added to this mixture and incubated with rotation for 30 min at room temperature. The tubes were then applied to a magnet (Dexter), the supernatant removed, and the beads were washed 3x with 1 mL PBS-T, mixing by inversion for each wash step. The proteins and bound aptamers were eluted from the Ni-NTA magnetic beads with 10 μL of 20 mM Tris, pH 7.5, 500 mM imidazole and transferred to PCR tubes. 100 μL PCR reactions contained 1.25 units Pfx polymerase

(Invitrogen), 1 μM primers "LICSelexF" and biotinylated "LICSelexR", 0.1 mM dNTPs, 0.5 mM MgSO4, and 0.1X enhancer solution. Amplification conditions were 2 min at 95 ^{o}C; 15 cycles: 30 sec at 95 ^{o}C; 30 sec at 56 ^{o}C; 30 sec at 68 ^{o}C; 2 min at 68 ^{o}C. This protocol produced 2-5 μg of the correct size product as determined using a DNA 500 lab-chip (Caliper) on an Agilent 2100 Bioanalyzer. After the amplification step, 90 μL of the PCR product and 23 μL 5M NaCl were then mixed with 1 mg of M-280 streptavidin magnetic beads (Dynal) for 10 min at room temperature, then washed 3x 1 mL with PBS-T. Single-stranded aptamers (non-biotinylated strand) were separated from the immobilized complimentary strand using a 5 min incubation of 50 μL of fresh 100 mM NaOH. The tubes were applied to a magnet and the ssDNA was removed and diluted into 1 mL PBS-T, containing 10 μL of 100 mM monobasic phosphate buffer to adjust the pH to 7.5. Finally, the material was heated to 95 ^{o}C for 2 min then immediately placed at 4 ^{o}C until the next round of SELEX.

For additional rounds of selection, the amount of protein was reduced to 50 pmol (rounds 2-10), and subsequently 25 pmol (rounds 11-15) in a binding volume of 1 mL, and the incubation time was reduced to 10 min After round 2, the PCR cycle number was reduced to 10 cycles because of the amplification of products of incorrect size. More than 15 cycles of amplification often led to the production of larger fragments, later identified as concatamers. In order to remove aptamers that bind to the Ni-NTA magnetic beads, counter-selection was performed after rounds 3, 6, 9, and 12. A 20 μL aliquot of a 5% slurry of Ni-NTA-magnetic beads was added to the 1 mL of ssDNA in PBS-T and incubated for 10 min with rotation, then applied to a magnet and the supernatant removed for the next round.

After round 15, the material was amplified by PCR with "LICSelex F" and "LICSelexR" primers, and the products were purified with MinElute (Qiagen), LIC-cloned into pET30XaLIC vector (Novagen), and transformed into NovaBlue *E. coli* (Novagen). 32 colonies were picked for each sample, and the plasmids purified by 96-well mini-prep (Qiagen). The plasmids were sequenced using a T7 promoter primer in the Big-dye Terminator kit and run on an ABI 3730. Sequences were aligned using ClustalX v.1.81 (19). Pattern analysis was performed using Consensus (20).

Aptamer-enzyme linked assay. To measure the binding of aptamers to proteins immobilized on microtiter plates, 500 ng of purified TTF1 or purified HOX4 fragment was bound to wells of a Ni-NTA HisSorb plate (Qiagen) in 200 μL PBS-T for 2 hours at room temperature. The wells were then washed 3x with 200 μL PBS-T. Biotinylated aptamers were diluted to 1 ng/μL in 200 μL PBS-T, heated to 95 ^{o}C and then cooled quickly to 4 ^{o}C. 200 μL of aptamer was incubated with proteins in the HisSorb plate overnight at 4 ^{o}C on a plate vortex shaking gently. The wells were washed 4x with 200 μL PBS-T for 5 min each on a plate vortex. Streptavidin-HRP (Molecular Probes) was diluted 1:10,000 into PBS-T and a 200 μL aliquot was incubated with the proteins and bound aptamers in the HisSorb plate for 30 min at room temperature. The wells were washed again as described above, then 150 μL of Turbo-TMB (Pierce) was added to each well and incubated for 20 min at room temperature in the dark. The reactions were stopped with the addition of 150 μL of 1 M H_2SO_4 and the protein bound aptamer-streptavidin complex was quantified by determining the absorbance at 450 nm using a SpectraMax Plus (Molecular Devices).

BIAcore surface plasmon resonance. The affinity of the aptamers for their protein targets was measured using surface plasmon resonance (SPR) with a BIAcore X instrument. Biotinylated aptamer (Operon) was diluted to 0.5 ng/μL in HBS-P (10mM HEPES, pH 7.5, 150 mM NaCl, 0.05% Tween 20), heated to 95 ^{o}C, and rapidly cooled at 4 ^{o}C before use. Approximately 100 RU of biotinylated aptamer (ligand) was immobilized to one flow cell of a streptavidin coated sensor chip. Purified TTF1 protein was diluted into HBS-P to give a series of concentrations of TTF1 protein (3, 12, 31, 62, 125, 250 nM or 2.5, 5, 10, 20, 40, 100 nM) that were injected over the surface for 2 min at a flow rate of 20 μL/min (to minimize mass transfer limitations). Bulk shift and non-specific interactions with the streptavidin were subtracted using the response from a reference flow cell. After measuring the off rates for 2 min for each analyte injection, complete regeneration of the surface was achieved with two 30 sec. injections of 0.05% SDS at 50 uL/min The affinity, as defined by the equilibrium dissociation constant (K_D), was determined globally by fitting to the kinetic simultaneous k_a/k_d model, assuming Langmuir (1:1) binding. The steady-state affinity was determined from curve-fitting to a plot of the Req values, derived from sensorgrams fitted locally, against the concentrations.

Protein blot analysis with aptamers. Protein samples were prepared for SDS-PAGE by boiling in Laemmli sample buffer and then resolved on denaturing 4-20% polyacrylamide gels using the mini-Protean 3 system (Bio-Rad). The proteins were either stained with Gelcode Blue (Pierce) or transferred to PVDF (Schleicher and Schuell). The PVDF membranes were blocked overnight at 4 ^{o}C with 5% BSA in PBS-T, and then probed with biotinylated aptamer diluted to 1 μg/mL in 5 mL PBS-T for 2 hours at room temperature with rotation. The blots were washed 3x for 5 min with 10 mL PBS-T and then probed with Streptavidin-HRP diluted 1:10000 in PBS-T. The blots were washed 3x for 5 min before chemiluminescence detection using pico-west substrate (Pierce). The blots were imaged using a Fluor-S Multi-Imager (Bio-Rad).

Aptamer affinity purification. Aptamers immobilized to magnetic beads were utilized for native protein purification. 10 μg of biotinylated aptamer was diluted into 200 μL PBS-T in a PCR tube and heated to 95 ^{o}C for 2 min, then immediately placed at 4 ^{o}C for 5 min. This material was added to 2 mg M-280 streptavidin magnetic beads (Dynal), and 50 μL of 5 M NaCl was added, and the mixed for 30 min with rotation at room temperature. In order to determine the level of non-specific binding to the M-280 beads, we performed the purification with biotin bound, instead of aptamer. The beads were washed 2x with 1 mL PBS-T before the purification. 100 μL of cleared lysate from the protein purification described above was spiked with 10 μg of partially purified target protein and then diluted 1:3 with PBS-T. The protein was eluted with DNAse treatment using 12 uL PBS-T containing 50 mM NaCl, 5 mM $MgCl_2$, and 60 units of Benzonase (Novagen). Several binding and elution schemes were tested: 1) 10 min binding at 4 ^{o}C and 2 hour nuclease treatment at 4 ^{o}C; 2) 30 min binding at 4 ^{o}C and 15 min nuclease treatment at room temperature; 3) 5 min binding and 5 min nuclease treatment at room temperature. For each set of conditions, the beads were washed 4x with 1 mL PBS-T containing 600 mM NaCl, and then washed 2x with 1 mL PBS-T containing 50 mM NaCl to adjust the ionic strength for optimal nuclease acitivity. Protein that remained after nuclease treatment was removed from the aptamer beads with 12 μL of

0.05% SDS. The samples were analyzed by SDS-PAGE on a 4-20% gel that was stained with GelCode blue (Pierce).

Results

Enrichment of aptamers to TTF1. We have optimized an *in vitro* ssDNA selection protocol that utilizes hexaHis-tagged protein targets bound to Ni-NTA magnetic beads to screen for high affinity binding aptamers from a library of approximately $6x10^{14}$ sequences. We used Ni-NTA magnetic beads to provide a universal support for His-tagged proteins as well as to facilitate the rapid partitioning of protein-aptamer complexes from unselected sequence pools. During the course of optimizing our protocol we had observed enrichment of sequences that were not unique to a particular protein, and therefore incorporated counter-selection steps against Ni-NTA magnetic beads to prevent enrichment of aptamers that recognize the beads only. The number of PCR cycles was also optimized to avoid overamplification, which is evidenced by larger products. The stringency of the selection was controlled by adjusting the target protein concentrations, the incubation times, and the washes. In order to assess our protocol, the enrichment of aptamers that bind TTF1 was monitored after 5, 10, and 15 rounds of selection. Multiple sequence alignments using ClustalX revealed that no enrichment was evident after 5 rounds of selection, however after 10 rounds of selection several groups of sequences were modestly enriched (2 or 3 of 32, not shown). Satisfactory enrichment was accomplished with 15 cylces of selection (Fig. 1). Five groups of identical sequences were identified after the 15 rounds of selection, including one aptamer “A” that represented 30% of the total evaluated. Therefore, our selection conditions using magnetic beads were sufficiently stringent for successful enrichment in 15 rounds. Interestingly, there were 2 aptamers “A” and “C” that contained a consensus sequence, aside from several G repeats found in each group (20). The TTF1 dsDNA binding consensus sequence (5’ T(C/T)AAGTG 3’) is not contained in the aptamer sequence. In addition to the aptamers described in Fig. 1, there were 3 sequences that were each represented once (not shown).

Determination of the specificity of a TTF1 aptamer. We used an enzyme-linked assay in order to prioritize the aptamers from the TTF1 selection for further characterization (not shown). This assay provides a rapid assessment of the relative binding capabilities of many aptamers from a particular selection experiment. In addition, the enzyme-linked assay was used to provide information regarding cross-reactivity (Fig. 2). Employing a colorimetric detection system (Turbo-TMB + sulfuric acid) for peroxidase activity conjugated to streptavidin, we observed a significant (100x) signal over background, and the data for triplicate samples ranged from 0.006 +/- 0.0002 to 0.63 +/- 0.05 absorbance units. In order to determine if the TTF1 aptamer ”A” would recognize another homeodomain family member, the enzyme-linked assay was used to show that the TTF1 aptamer “A” does not cross-react with the homeodomain of HOX4 (Fig. 2), nor did the aptamer bind BSA. In addition, an aptamer that was selected for HOX4 binding (not described here) did not cross-react with the TTF1 protein.

Determination of the affinity of aptamers for TTF1. We utilized surface plasmon resonance employing a BIAcore X instrument to measure the affinity of the interaction of TTF1 with aptamers immobilized on a sensor chip. Sensorgrams of a concentration series of TTF1 injected over aptamers “A” or “C” are shown in Fig. 3, A

and C, respectively. The affinity, K_D, was determined by a global fit using the kinetic simultaneous k_a/k_d model, assuming Langmuir (1:1) binding. The affinity of aptamer "A" for TTF1 was 3.36 x 10^{-9} M, and the affinity of aptamer "C" for TTF1 was 3.25 x 10^{-8} M. The steady state affinities of TTF1 for the aptamers, determined from plots of Req values derived from sensograms in (A and C) fitted locally, correlated well with the simultaneous k_a/k_d model (Fig. 3, B and D).

Comparison of the specificity of the TTF aptamer to a monoclonal anti-Penta-His antibody using protein blot analysis. The results of the enzyme-linked assay suggested that the TTF1 aptamer "A" exhibited specificity for TTF1. In order to verify the specificity and determine whether the aptamer recognized the denatured form of TTF1, as well as to investigate further the potential uses of the aptamer, we performed a protein blot analysis (Fig. 4). The TTF1 aptamer "A" was indeed able to bind the denatured TTF1 on the blot (Fig. 4 C lanes 3, 4) and exhibited very little non-specific binding to the proteins in the cleared *E. coli* lysate or to the purified HOX4 (Fig. 4 C lanes 1, 2). The performance of the aptamer was similar to the anti-PentaHis antibody in terms of chemiluminescent signal intensity and specificity (Fig. 4 B). Note that the bands in lane 4 of B and C below the major TTF1 band (marked with an arrow) are degradation products of TTF1 as determined by MALDI mass spectromic analysis (not shown). Also, there is an approximately 20 kDa protein in the *E. coli* lysate (Fig. 4 C lanes 1, 3) that is recognized by the streptavidin-HRP secondary and not due to cross-reactivity of the aptamer (not shown).

Aptamer affinity purification. We performed aptamer affinity chromatography from a complex mixture of proteins in the soluble fraction of bacterial lysates using biotinylated aptamers on streptavidin magnetic beads (Fig. 5). TTF1 aptamer "A" specifically purified the recombinant TTF1 protein out of the *E. coli* lysate in a single purification step. Elution of all proteins bound to the aptamer "A" magnetic beads with SDS, which removes all bound proteins from the beads, showed that the purification of TTF1 was highly specific. We then tested generic elution conditions that would be most amenable to high-throughput methods. Elution of the purified TTF1 from the affinity matrix was inefficient with 1 M NaCl (not shown), therefore we tested elution with a recombinant DNase. The recovery of purified TTF1 with DNase treatment (lanes 4, 6, 8) was approximately 25-50% of the total protein bound to the affinity column as revealed by a subsequent denaturing elution with SDS (lanes 5, 7, 9). The efficiency of elution with DNase was better when the affinity beads were not saturated with TTF1 protein. This is likely due to the accessibility of the aptamer, which may be protected in conditions of saturating amounts of TTF1 protein. Additional optimization for improved elution yield of specific proteins without denaturing could be investigated on a case by case basis. This work further illustrates the potential utility of aptamers and demonstrates single-step purification from bacterial lysates.

Discussion

We have developed an improved and straightforward protocol for DNA aptamer production and have characterized aptamers that recognize thyroid transcription factor 1 (TTF1), a member of the NK homeodomain transcription factors (15). The use of aptamers as protein affinity reagents offers advantages over the use of antibodies. Nucleic acids are easily synthesized or amplified by PCR, therefore a vast supply of

consistent quality is available. Also, nucleic acids can easily be modified to incorporate tags, such as biotin or fluorescent molecules, for detection and/or immobilization. Additionally, aptamers are smaller (< 25 kDa) and more stable than antibodies. Moreover, unlike the requirement of milligram quantities of protein or peptide for antibody production, only microgram quantities of protein or peptide are required for aptamer SELEX. These properties, coupled to the present technology available for DNA microarrays, make aptamers very suitable for use in protein microarrays as a ligand, or for detecting proteins bound to a chip surface (21).

Despite these advantages, aptamers have rarely been selected for general use since the technology was developed 13 years ago. Approximately 40 unique aptamers against proteins or peptides have been described in more than 300 references in the literature. This lack of widespread use may be attributed to challenges in adapting existing protocols to particular targets and a general lack of fine details in existing methods. Many variations in aptamer production protocols have been described in which the method of protein target partitioning seems to vary the most. Unselected aptamers have been removed from target proteins via: 1) filtration on a membrane (13); 2) column chromatography, in which the targets are bound to a matrix, such as sepharose, using a covalent linkage or an affinity tag (22); 3) binding of the protein to the wells of a microtiter plate (23).

The novelty of the protocol that we have described is the use of Ni-NTA magnetic beads for the immobilization of His-tagged protein targets during selection. Hexahistidine tags are widely used in recombinant protein production, for example our group has described an efficient protein production pipeline for high-throughput generation of His-tagged proteins in *E. coli*. (4). The use of a tag for immobilization promotes the proper orientation of proteins uniformly on a bead surface, and simultaneously provides a purification step, thereby reducing the chances of selection toward contaminants. Paramagnetic beads are an optimal solid support for parallel processing of both proteins and nucleic acids. Very small amounts of magnetic beads with proteins bound can be rapidly partitioned from unselected material, stringently washed, and subsequently eluted. Others have claimed that filtration is necessary to partition the bound aptamers from unselected ones in order to obtain sufficient stringency (14). However, in our protocol the washes were sufficiently stringent using magnetic beads. This is an advantage because partitioning by filtration is a cumbersome process for multiple targets but magnetic bead separations are easily accomplished in parallel, manually or automated. Additionally, we have taken advantage of the highly specific and strong streptavidin-biotin interaction for several applications: 1) generating single stranded material from biotiylated PCR products after amplification at each round of selection using streptavidin magnetic beads; 2) detecting biotinylated aptamers in enzyme-linked assays and western blot analysis; 3) immobilizing biotinylated aptamers to streptavidin beads for purification of protein target; 4) immobilizing biotinylated aptamers to streptavidin sensor chips in BIAcore measurements.

Using manual processing (by one person) we were able to complete 3 rounds of selection per day on 8 samples. We found that 15 rounds of selection produced high affinity aptamers. Therefore our present throughput could be approximately 32 aptamers per month. However, our protocol is amenable to a 96-well approach and

could be scaled-up to produce about 384 aptamers per month using manual processing. Others have described an automated aptamer acquisition platform with a throughput of 120/month for 8 proteins, however it requires customized robotics not available to many labs (14). Another high throughput SELEX protocol using 96-well microtiter plates has been described that is compatible with robotics and was tested manually (23). However, that protocol relied on hydrophobic immobilization of proteins on microtiter plates, and the authors concede that the four proteins tested adhered to the wells with varying efficiency, making it difficult to control the amount of protein in each experiment. In addition, use of a hydrophobic immobilization would also result in proteins with variable orientations on the surface, reducing the effective concentration of available active sites. Lastly, since this method is nonspecific, it would also result in the immobilization of all proteins in the sample, including protein contaminants that could interfere with the aptamer selection process.

We have illustrated the functional versatility of aptamers in several assays. Enzyme-linked assays provide a means of quickly evaluating a group of aptamers from a selection by measuring their relative affinities, and this kind of triage can be used to prioritize aptamers for more detailed characterization (24). We demonstrate that enzyme-linked assays can also provide information about cross-reactivity. We are able to measure significant signals over very low background by using biotinylated aptamers to detect proteins in a 96-well microtiter plate and a peroxidase conjugated streptavidin and colorimetric substrate to detect the bound aptamers. Enzyme-linked assays offer advantages over other techniques, such as equilibrium dialysis and electrophoretic-mobility shift assays, that are used to evaluate aptamers from a selection. These advantages include the lack of radioisotope usage, increased throughput in a 96-well plate, minimization of waste, and ease of precise quantitation of the relative binding affinities.

Using aptamers in a protein blot analysis is another means of characterizing their specificity. We have tested several aptamers in chemiluminescent protein blot analysis, however only one of the TTF1 aptamers worked in this application suggesting that, just as some antibodies fail to recognize the denatured form of a protein, some aptamers will recognize epitopes that are absent in the denatured form of the protein. The TTF1 aptamer showed no cross-reactivity to *E. coli* proteins in a cleared lysate on the blot and was similar in specificity observed for the anti-PentaHis-HRP antibody.

The key function of high affinity aptamers in applications such as protein purification, protein profiling chips, and diagnostics is to recognize and separate the target protein from a complex mixture of proteins. We have described in this work the first successful application of aptamer affinity chromatography for one-step purification of a protein from the complex mixture of proteins in the soluble fraction of bacterial cell lysates. Although aptamer affinity chromatography has been described and demonstrated for the purification of a protein from conditioned cell culture media, this purification technique has not been previously demonstrated for more complex samples such as cell lysates (25). Detrimental effects from DNase activity in our purification from bacterial lysates were not observed, unlike the problems associated with DNase degradation of aptamers that occurs when purifying targets from serum (25). Importantly, aptamer affinity chromatography provides a means of protein purification of

the native form of a protein without relying on affinity tags that may adversely affect protein structure, function or ability to form crystals for structural characterization.

We have recently learned that TTF1 is a highly specific marker for primary lung adenocarcinomas, and antibodies against TTF1 have been recommended to be included in a panel of antibodies for the differential diagnosis between primary and metastatic adenocarcinomas of the lung (26). We have initiated studies in our lab to determine whether or not the aptamers that we have produced against *Ciona intestinalis* TTF1 recognize human TTF1, if so the aptamers described here may become a valuable diagnostic tool for primary lung adenocarcinoma.

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Figure Legends

Fig. 1 Enrichment of aptamers to TTF1 from 15 rounds of selection. The 40 base variable region of each aptamer sequence is shown (5'-3'). The number of times that each sequence was obtained from a total of 30 isolates is displayed on the right. A consensus sequence found in two of the aptamers is underlined.

Fig. 2. Determination of specificity of aptamers using an enzyme-linked assay. Combinations of TTF1, HOX4, and BSA protein and either a TTF1 aptamer or HOX4 aptamer (indicated by plus signs below the graph) were evaluated for their binding activity and cross-reactivity. The data are from triplicate samples.

Fig. 3. Determination of the affinities of aptamers for TTF1 using surface plasmon resonance. (A) Sensorgrams of the binding response to aptamer "A" measured for concentrations of 2.5, 5, 10, 20, 40, 100 nM TTF1 analyte. The K_D=3.36x10-9 M as determined from a global fit of the kinetic simultaneous k_a/k_d model, assuming Langmuir (1:1) binding, and Chi^2= 14.1. (B) Plot of the steady-state affinity for "A" using the Req values derived from sensorgrams in (A) fitted locally. The K_D=5.14x10-9 M as determined from the steady state affinity model. (C) Sensorgrams of the binding responses to aptamer "C" measured for concentrations of 3, 12, 31, 62.5, 125, 250 nM TTF1 analyte. The K_D=3.25x10-8 M as determined from a global fit of the kinetic simultaneous k_a/k_d model, assuming Langmuir (1:1) binding, and Chi^2=10.9. (D) Plot of the steady-state affinity for "C" using the Req values derived from sensorgrams in (C) fitted locally. The K_D=6.56x10-8 M as determined from the steady state affinity model.

Fig. 4. Comparison of the specificity of the TTF aptamer to a monoclonal anti-Penta-His antibody in a protein blot analysis. Lane 1 contains cleared lysate from *E.coli* expressing the HOX4 homeodomain. Lane 2 contains purified HOX4 homeodomain protein (marked with an arrow). Lane 3 contains cleared lysate from *E.coli* expressing TTF1. Lane 4 contains purified TTF1 protein (marked with an arrow). (A) 4-20% SDS-PAGE stained with GelCode blue. (B) Blot of material shown in (A) probed with an anti-PentaHis monoclonal antibody. (C) Blot of material shown in (A) probed with the biotinylated TTF aptamer "A". Note that the lower dark bands in lanes 1 and 3 of (C) were detected by the secondary probe, Streptavidin-HRP, (not shown).

Fig. 5. SDS-PAGE analysis of aptamer affinity purification of TTF1 protein from *E.coli* lysates using biotinylated aptamers immobilized on streptavidin magnetic beads. The gel is 4-20% polyacrylamide stained with GelCode blue. Lane 1 contains cleared lysate from *E.coli* expressing the protein of interest and lane 2 contains the cleared lysate spiked with Ni-NTA purified TTF1 protein (lane 3). Material in lane 4 was from 10 min binding and 2 hr. elution at 4°C. Material in lane 6 was from 30 min binding and 15 min elution at room temperature. Material in lane 8 was from 5 min binding and 5 min elution at room temperature. After each elution with Benzonase, any remaining protein was removed from the aptamer with 0.1% SDS (lanes 5, 7, 9).

Figure 1

Aptamer	Variable Sequence	Enrichment
A	tcaaaaggggtgattgcttgcacaatgacagggtaggaca	9
B	gatacacgggcggaggaggtgggggggggtaggtgggtat	7
C	tggctagtgggtaaggggcgggagggtgacagggcgatcc	6
D	ttatggggatgaaagtggtgttcgggttcgccacttccac	3
E	ttggggtgggagggcgggttaacaaagatagcgcaacagg	2

Figure 2

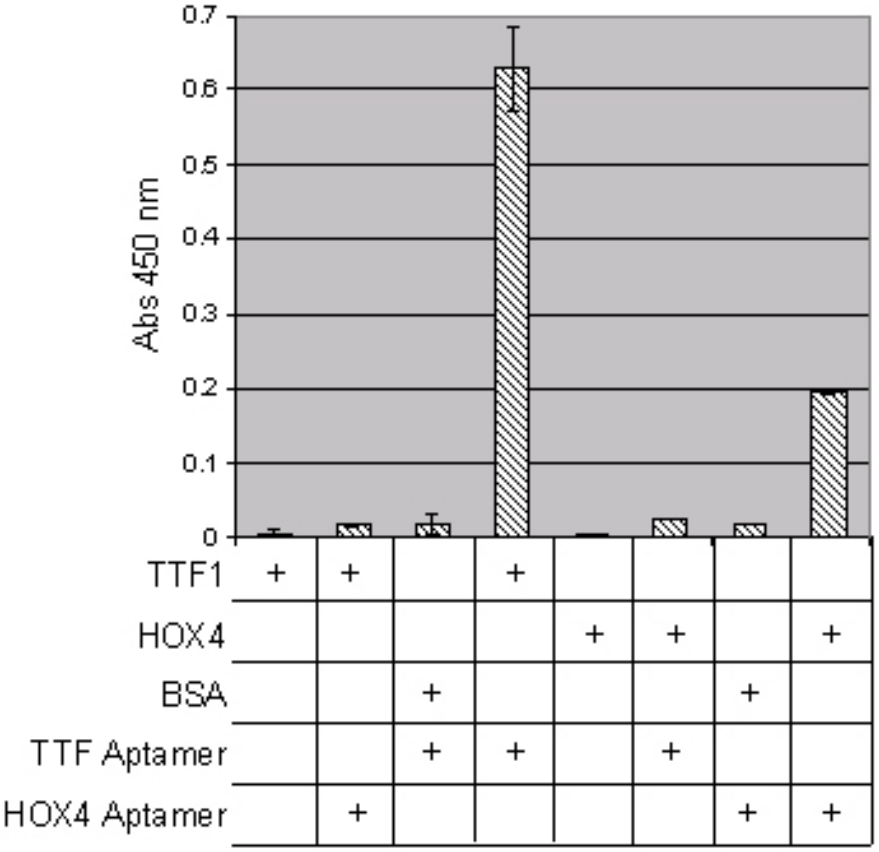

Figure 3

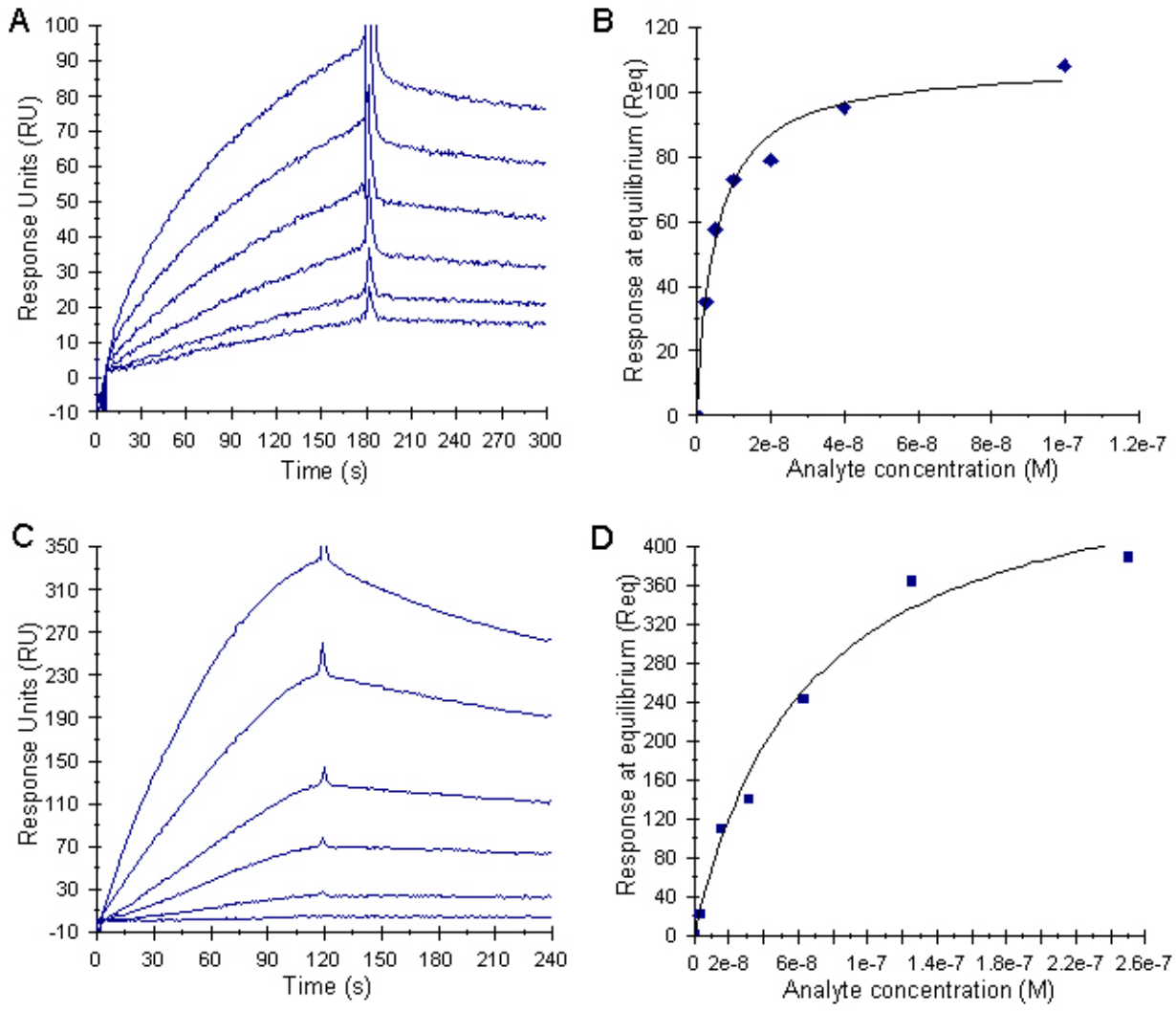
A
Response Units (RU)
Time (s)
B
Response at equilibrium (Req)
Analyte concentration (M)
C
Response Units (RU)
Time (s)
D
Response at equilibrium (Req)
Analyte concentration (M)

Figure 4

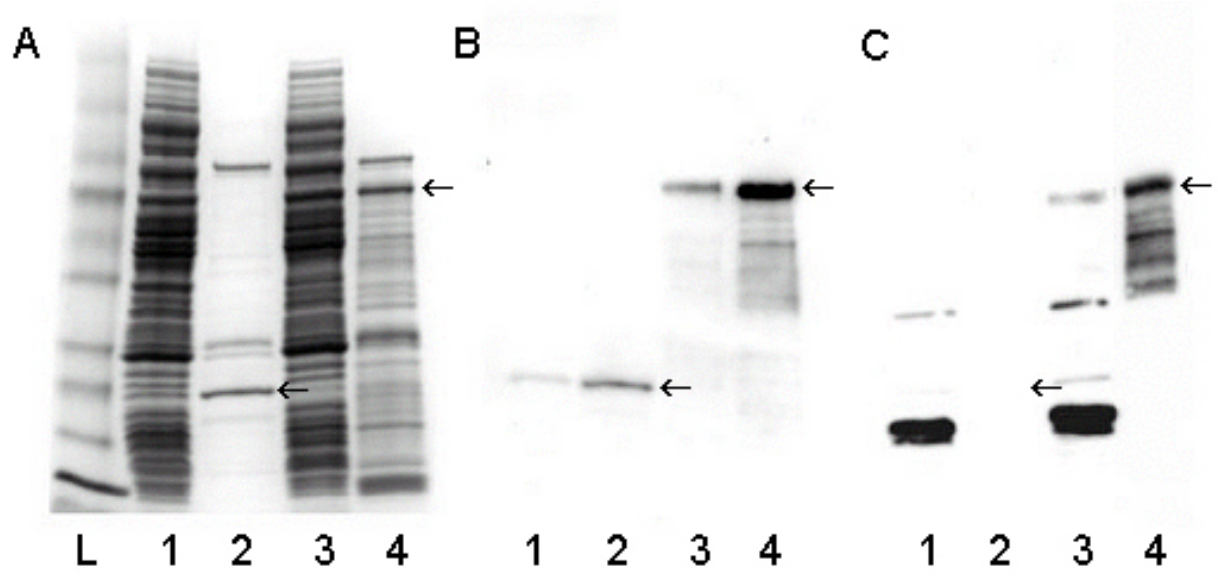

Figure 5